Attenuation of Replication-Competent Adenovirus Serotype 26 Vaccines by Vectorization

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Replication-competent adenovirus (rcAd)-based vaccine vectors may theoretically provide immunological advantages over replication-incompetent Ad vectors, but they also raise additional potential clinical and regulatory issues. We produced replication-competent Ad serotype 26 (rcAd26) vectors by adding the E1 region back into a replication-incompetent Ad26 vector backbone with the E3 or E3/E4 regions deleted. We assessed the effect of vectorization on the replicative capacity of the rcAd26 vaccines. Attenuation occurred in a stepwise fashion, with E3 deletion, E4 deletion, and human immunodeficiency virus type 1 (HIV-1) envelope (Env) gene insertion all contributing to reduced replicative capacity compared to that with the wild-type Ad26 vector. The rcAd26 vector with E3 and E4 deleted and containing the Env transgene exhibited 2.7- to 4.4-log-lower replicative capacity than that of the wild-type Ad26 *in vitro*. This rcAd26 vector is currently being evaluated in a phase 1 clinical trial. Attenuation as a result of vectorization and transgene insertion has implications for the clinical development of replication-competent vaccine vectors.

The development of a safe and effective human immunodeficiency virus type 1 (HIV-1) vaccine is an urgent global health priority. An HIV-1 vaccine should be simple to administer, be long acting, and provide protection against mucosal HIV-1 exposure. Replication-incompetent adenovirus serotype 26 (Ad26) has shown promise as a candidate HIV-1 vaccine vector. Preclinical studies in rhesus monkeys have shown that replication-incompetent Ad26 vector-based vaccine regimens can provide partial protection against both simian immunodeficiency virus 251 (SIVmac251) and simian-human immunodeficiency virus (SHIV)-SF162P3 challenges (1, 2). In addition, a replication-incompetent Ad26 vector expressing the HIV-1 envelope (Env) gene proved safe and immunogenic in phase 1 clinical trials (3–5). A related strategy is to use live replicating vaccine vectors that express HIV-1 antigens (6). We therefore explored the potential utility of a replicating Ad26 vector as a candidate HIV-1 vaccine.

Mosaic HIV-1 Env immunogens (e.g., Mos1Env) were developed to begin to address the challenge of HIV-1 sequence diversity (7). Mosaic immunogens are bioinformatically engineered to optimize coverage of global HIV-1 sequence diversity (7–12). Mosaic HIV-1 immunogens have shown increased breadth and depth of cellular immune responses compared with those of consensus or natural sequences in monkeys (7, 11). It has also been shown that adenovirus and poxvirus vectors expressing mosaic Gag, Pol, and Env sequences afforded a significant reduction in the per-exposure risk of SHIV-SF162P3 acquisition in rhesus monkeys (2). Moreover, mosaic Env antigens induced greater binding and neutralizing antibodies than those of natural sequence antigens (7).

A replication-competent Ad vector might provide several theoretical advantages over a replication-incompetent vector, including the potential for improved cellular and humoral immune responses and augmented mucosal effector responses. In this study, we describe the preclinical development of a replication-competent Ad26 (rcAd26) vaccine vector that expresses mosaic HIV-1 Env. We assessed the impact of vectorization on the growth kinetics of rcAd26 *in vitro*. Specifically, we evaluated whether the deletion of E3 and/or E4 and insertion of the HIV-1 Env transgene would result in vector attenuation. In addition, we compared the replicative capacity of rcAd26 to an rcAd4 vector that has already been shown to be safe in phase 1 clinical trials (13–15) to facilitate first-in-human clinical testing of the rcAd26 vector.

MATERIALS AND METHODS

Vector construction. The rcAd26 vector system was produced by cloning the adenovirus E1 region from wild-type Ad26 into pAdApt26, the adaptor plasmid that we had previously made to produce replication-incompetent Ad26 vectors (16). The adaptor plasmid for the rcAd vector system, termed pAdApt26.E1atg, has a final structure that includes the left inverted terminal repeat of Ad26 nucleotides 1 to 471 flanked by the transgene cassette under the control of a cytomegalovirus (CMV) promoter and SV40 poly(A), followed by the Ad26 nucleotides 463 to 5913 cloned into a pBR322 backbone. This adapter plasmid contains sufficient Ad26 sequences to allow for homologous recombination with an Ad26-derived cosmid after cotransfection. The Ad5 E4orf6 sequences in the pWe.Ad26.dE3.5orf6 cosmid (16) were also replaced with the Ad26 E4orf6 sequences from wild-type Ad26. To accommodate large transgene sequences, the E4orf1 to E4orf4 regions (nucleotides 33261

Received 8 September 2015 **Accepted** 9 September 2015

Accepted manuscript posted online 16 September 2015

Citation Maxfield LF, Abbink P, Stephenson KE, Borducchi EN, Ng'ang'a D, Kirilova MM, Paulino N, Boyd M, Shabram P, Ruan Q, Patel M, Barouch DH. 2015. Attenuation of replication-competent adenovirus serotype 26 vaccines by vectorization. Clin Vaccine Immunol 22:1166–1175. doi:10.1128/CVI.00510-15.

Editor: H. F. Staats

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to 34683), in addition to E3, were deleted. All plasmids and cosmids were screened by restriction enzyme analysis and complete sequencing. The rcAd26.dE3.dE4.Mos1Env vector was also fully sequenced. The rcAd4.H5 (Ad4-H5-Vtn) vector and Ad4-wild type (WT) were obtained from PaxVax (San Diego, CA).

Vector production. Vectors were produced as described previously (16, 17). Briefly, Ad26-based vectors were produced by homologous recombination after cotransfection of an Ad26 adaptor plasmid that expressed either no transgene (empty) or HIV-1 Mos1Env, along with the appropriate Ad26 cosmid with either E3 or E3/E4 deleted. The plasmids and cosmids were linearized prior to transfection of Per55K cells with Lipofectamine 2000 (Invitrogen) in T25 flasks. After 48 h, cells were passaged in T75 flasks and monitored for virus cytopathic effect. The vectors were plaque purified, and the plaques were screened for transgene sequence and expression and by PCR to confirm the hexon sequences. Vectors were then produced in 24 triple-layer flasks, purified by a cesium chloride gradient ultracentrifugation, and dialyzed into phosphate-buffered saline (PBS) containing 5% sucrose. Purified Ad26 vectors were stored at −80°C. Virus particle (vp) titers were determined by spectrophotometry, and specific infectivity was assessed by PFU assays.

***In vitro* replicative capacity.** The growth of a panel of rcAd26 vectors was compared to that of wild-type Ad26 in the following cell lines: A549 (human epithelial lung carcinoma cell line, ATCC CCL-185; ATCC, Manassas, VA), HuTu80 (human duodenum adenocarcinoma cell line; ATCC HTB-40), Per55K (human cell line that complements the Ad E1 region), and LLC-MK2 (rhesus kidney cell line, ATCC CCL-7). Each cell line was grown in Dulbecco's modified Eagle medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Seradigm, UT, USA). PFU assays were performed by infecting A549 cells that had been seeded into 6-well plates at a concentration of 8.5×10^5 cells per well the day before with serial dilutions of adenovirus. The next day, an agar overlay was added, and plaques were counted at days 7 and 14 postinfection. All serial dilutions were done in duplicate.

The kinetics of virus replication was also assessed in a semiquantitative cytopathic effect (CPE) assay: the day before infection, cells were seeded into 6-well plates at a concentration of 8.5×10^5 cells per well. The next day, cells were infected with each adenovirus vector at multiplicities of infection (MOIs) of 1,000, 333, 100, 33, 10, and 0 vp per cell. The number of virus particles per milliliter of the starting material was determined by the optical density (OD), and the virus particle-to-PFU ratios for all of the vectors were similar (21 to 41 vp/PFU). The cultures were then monitored and scored daily for percent CPE for 6 days postinfection. Each vector and cell line combination was tested in 3 replicate experiments.

To confirm the infectivity of the viruses with two subsequent passages, viral lysates were harvested from each primary culture at full CPE by harvesting both the cells and supernatant by pipette and freezing at −20°C until further testing. Subsequent reinfections were performed by thawing and then clarifying each lysate by centrifugation. To reinfect the cells, 100 μl of each lysate was added to cells that had been seeded the day before at 8.5×10^5 cell per well into a 6-well plate. Cultures were then monitored and scored daily for percent CPE for 6 days postinfection and infected cell lysates harvested the day of full CPE.

Immunogenicity in mice. To assess the immunogenicity of the clinical candidate rcAd26.Mos1Env vector, BALB/c mice ($n = 4$) were immunized intramuscularly or intranasally with 1×10^{10} vp replication-competent rcAd26.dE3.dE4.Mos1Env or replication-incompetent (ri) vector ri(E1$^-$).Ad26.dE3.Mos1Env at day 0. Serum was obtained at day 0 preimmunization and weekly at days 7, 14, 21, and 28 postimmunization. Env-specific antibody titers in these sera were assessed by enzyme-linked immunosorbent assay (ELISA) to HIV-1 Mos1Env protein developed with SureBlue 3,3′,5,5′-tetramethylbenzidine (TMB) Microwell peroxidase substrate (KPL). Log_{10} values were plotted using GraphPad Prism 6. Env-specific T lymphocyte responses were assessed by gamma interferon (IFN-γ) enzyme-linked immunosorbent spot assay (ELISPOT), as described previously (18, 19). Spleens were harvested at day 28 postimmunization, and splenocytes were isolated and stimulated with Mos1Env 1, Mos1Env 2, potential T cell epitope (PTE) Env 1, PTE Env 2, and PTE Env 3 peptide pools (20).

RESULTS

Construction of the rcAd26 vector system. A replication-competent version of Ad26 (rcAd26) was constructed by adding the Ad26 E1 region 5′ of the transgene cassette in the replication-incompetent version of our Ad26 vector (16). In addition, rcAd26 has the E3 region deleted and the Ad5 E4orf6 region replaced by the Ad26 E4orf6 to make the vector fully replication competent. The vector expressed the mosaic HIV-1 Env immunogen (Mos1Env) antigen. To ensure adequate coding capacity, we also deleted the E4orf1 to E4orf4 regions from rcAd26, resulting in our clinical candidate vector rcAd26.dE3.dE4.Mos1Env (Fig. 1A).

We first confirmed that rcAd26 could replicate in A549 cells, which do not complement the Ad E1 region. The growth kinetics of rcAd26.empty (with no transgene) was compared to that of wild-type Ad26 and the replication-incompetent Ad26 vector ri(E1$^-$)Ad26.dE3.empty. In this experiment, wild-type Ad26 and rcAd26.dE3.empty grew efficiently in A549 cells infected with an MOI of 1,000 or 100, whereas the replication-incompetent Ad26 vector did not. As expected, all vectors grew well in Per55K cells, which complements Ad E1 (Fig. 1B). Replication of the rcAd26 vectors was species specific, as these vectors did not replicate in the rhesus monkey cell line MK2 (Fig. 1C), whereas a replication-competent simian Ad vector derived from rhesus monkeys (21) replicated well in MK-2 cells. The rcAd26 vectors also did not grow in mouse cell lines (data not shown).

Replication-competent rcAd26 vectors are attenuated compared to wild-type Ad26. The infectivity of a panel of rcAd26 vectors (Table 1) was assessed in human cell lines to determine if the deletion of E3 and E4 and the insertion of the Mos1Env transgene impacted the viral replicative capacity *in vitro*. The rcAd26 vectors used in this study included wild-type Ad26, the clinical candidate rcAd26.dE3.dE4.Mos1Env, and other versions of the vector that had E3 and/or E4 deleted and were with or without the Mos1Env transgene. We utilized a quantitative PFU assay to evaluate virus titers, and we also evaluated virus growth kinetics utilizing semiquantitative CPE monitoring.

We first tested the replication capacity of these vectors by infecting HuTu80 (duodenal) or A549 (human epithelial) cells at an MOI of 1,000 vp/cell and harvesting cell lysates at full CPE. The viral titer in each lysate was quantitated by PFU assays in duplicate (Fig. 2). When the vectors were grown in HuTu80 cells, the rcAd26.dE3.empty vector had a mean PFU titer that was 1.3 logs lower than that of wild-type Ad26. However, with E3 and E4 deleted, the rcAd26.dE3.dE4.empty vector had a mean PFU titer 2.2 logs lower than that of wild-type Ad26. With the addition of the Env transgene, the mean PFU titer was 3.0 logs less than that of wild-type Ad26 when only E3 was deleted (rcAd26.dE3.Mos1Env). With both E3 and E4 deleted, the clinical candidate vector rcAd26.dE3.dE4.Mos1Env demonstrated the most pronounced attenuation, with a mean PFU titer 4.4 logs lower than that of wild-type Ad26 (Fig. 2; $P < 0.05$ for PFU titers of all recombinant vectors compared with wild-type Ad26). An attenuated replicative capacity was similarly observed in A549 cells. The rcAd26.dE3.empty vector had a PFU titer similar to that of wild-type Ad26. With E3 and E4 deleted, the rcAd26.dE3.dE4.empty vector had a PFU titer 1.9 logs lower than that

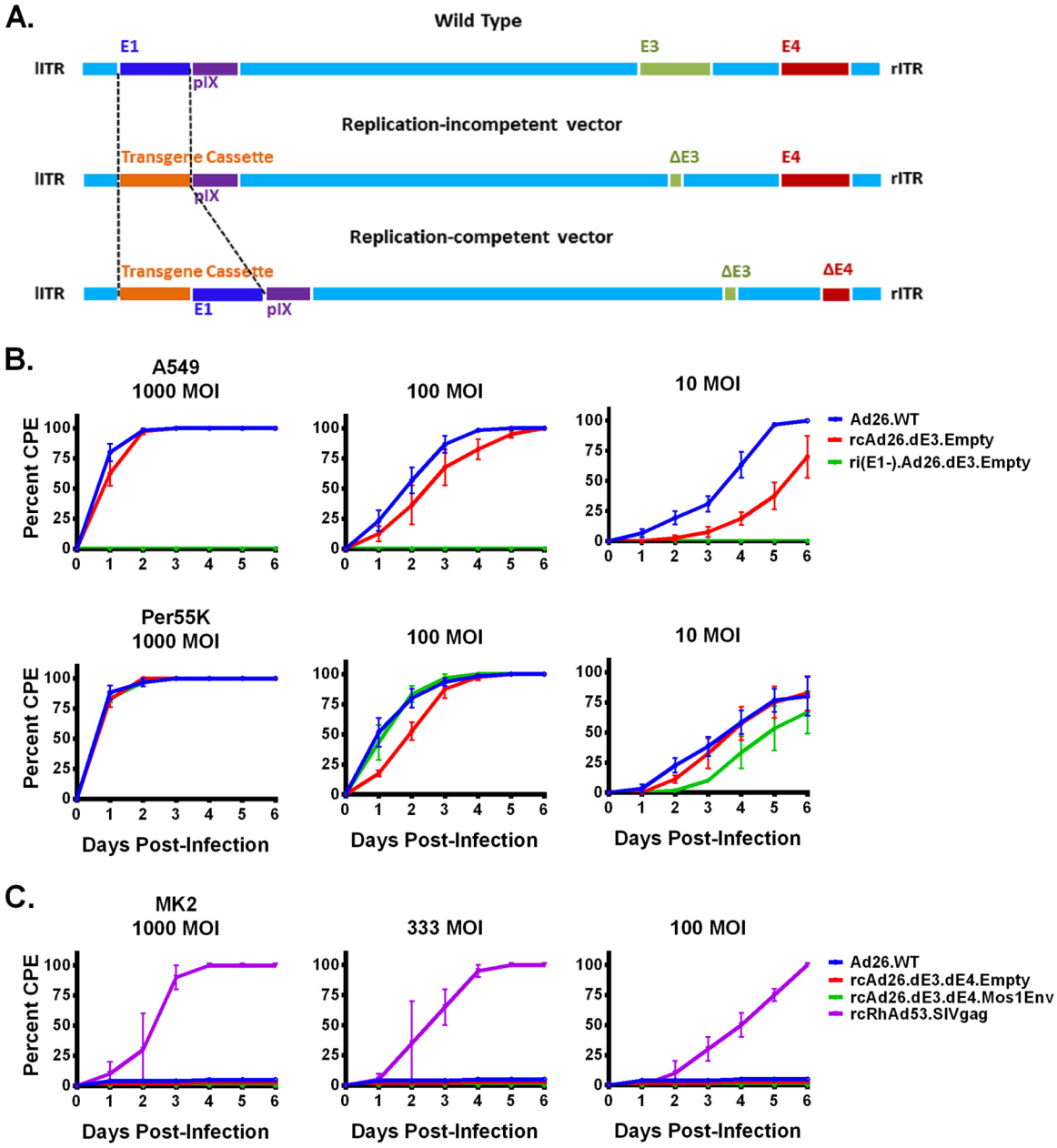

FIG 1 The rcAd26 vaccine vector replicates in human cell lines but not in a rhesus cell line. (A) Schematic of the construction of the replication-competent Ad26 vaccine vector from wild-type Ad26. lITR, left inverted terminal repeat; rITR, right inverted terminal repeat. (B) Vector replication was evaluated *in vitro* in A549 (human; does not complement E1) and Per55K (human; complements E1) cell lines. Cells were infected at MOIs of 1,000, 100, and 10, and they were monitored daily for CPE over 6 days. The rcAd26 vector growth was compared to that of the replication-incompetent vector ri(E1$^-$).Ad26.dE3 and wild-type Ad26. (C) Vector replication was evaluated *in vitro* in the MK-2 cell line (rhesus monkey kidney). Cells were infected at MOIs of 1,000, 333, and 100 vp/cell, and they were monitored daily for CPE over 6 days. Growth of a replication-competent simian adenovirus vector was utilized as a positive control. Error bars indicate standard errors of the means (SEM).

of wild-type Ad26. When the Mos1Env transgene was added, the rcAd26.dE3.Mos1Env vector had a PFU titer 2.1 logs lower than that of wild-type Ad26, and the rcAd26.dE3.dE4.Mos1Env vector had a PFU titer 2.7 logs lower than that of wild-type Ad26. These effects were not due to differential specific infectivities of the different vectors, since the virus particle-to-PFU ratios for all the recombinant vectors were similar (23 to 41 vp/PFU), which would not account for the 2.7- to 4.4-log difference in replicative capacity of the clinical candidate rcAd26.dE3.dE4.Mos1Env vector compared with the wild-type Ad26 vector.

In addition to the quantitative PFU assay, we also compiled semiquantitative vector growth kinetics in A549, HuTu80, and Per55K cells, expressed as percent CPE over time. All rcAd26 vectors exhibited slower growth kinetics than that of wild-type Ad26 in the A549 (human epithelial) (Fig. 3A) and HuTu80 (duodenal) (Fig. 3B) cell lines. Consistent with the quantitative PFU assay, the

TABLE 1 Panel of vectors used for *in vitro* infectivity comparison

Vector	Replication competent	E3 deleted	E4 deleted[a]	Transgene
rcAd26.dE3.dE4.Mos1Env[b]	Yes	Yes	Yes	HIV-1 Mos1Env
rcAd26.dE3.Mos1Env	Yes	Yes	No	HIV-1 Mos1Env
rcAd26.dE3.dE4.empty	Yes	Yes	Yes	No
rcAd26.dE3.empty	Yes	Yes	No	No
Ad26.WT	Yes	No	No	No
ri(E1$^-$)Ad26.dE3.empty	No	Yes	No	No
ri(E1$^-$)Ad26.dE3.Mos1Env	No	Yes	No	HIV-1 Mos1Env
Ad4.WT (PaxVax)	Yes	No	No	No
rcAd4.H5 (PaxVax)	Yes	Partial	No	Influenza H5

[a] E4orf1 to E4orf4 deleted.
[b] Clinical candidate rcAd26.Mos1Env.

semiquantitative growth kinetics suggested stepwise reductions with E3 deletion, E4 deletion, and HIV-1 Env transgene insertion. The clinical candidate vector rcAd26.dE3.dE4.Mos1Env demonstrated substantial reductions in growth kinetics compared with that of wild-type Ad26 in both cell lines. As expected, the replication-incompetent Ad26 vectors did not replicate in either the A549 or HuTu80 cell line. As a control, we observed that all vectors replicated well in E1-complementing Per55K cells (Fig. 3C).

To verify that the CPE observed in this assay system indicated that the rcAd26 was an infectious virus, we harvested cultures at full CPE and performed two additional passages of the virus in either A549 or HuTu80 cells. One hundred microliters of cell lysate harvested at full CPE from the cultures infected with either 1,000 vp/cell or 333 vp/cell of each vector was used for reinfection. Lysates harvested from the first reinfection at full CPE were then used for the second reinfection. All rcAd26 vectors replicated through 2 passages in both A549 and HuTu80 cell lines, indicating that the CPE observed reflected infectious virus and not nonspecific cell toxicity (Fig. 4A and B).

rcAd26.dE3.dE4.Mos1Env is more attenuated than rcAd4.H5. We next compared the *in vitro* growth kinetics of our clinical candidate vector, rcAd26.dE3.dE4.Mos1Env, to that of rcAd4.H5 (Ad4-H5-Vtn; PaxVax), which is an Ad4-based vector expressing influenza H5 and was previously proven in a phase 1 clinical trial to be safe and immunogenic (13–15). The rcAd26.dE3.dE4.Mos1Env vaccine showed markedly reduced replicative capacity compared to that of rcAd4.H5 in both A549 cells (Fig. 5A) and HuTu80 cells (Fig. 5B). As expected, all vectors grew well in Per55K cells (Fig. 5C).

rcAd26.dE3.dE4.Mos1Env induces humoral and cellular immunity in mice. To verify that Mos1Env was immunogenic when expressed from the clinical candidate rcAd26.dE3.dE4.Mos1Env, BALB/c mice ($n = 4$) were immunized either intramuscularly or intranasally with a single administration of 1×10^{10} vp of the clinical candidate rcAd26.dE3.dE4.Mos1Env vector or replication-incompetent vector ri(E1$^-$).Ad26.dE3.Mos1Env. However, rcAd26 vectors do not replicate in mice (data not shown); thus, this experiment was unable to assess the impact of vector replication on immune responses. The antibody titers in sera from day 0 preimmunization and day 28 postimmunization were assessed by ELISA. As shown in Fig. 6A, rcAd26.dE3.dE4.Mos1Env induced binding antibodies to MosI Env glycoprotein 140 (gp140) antigens. The titers were comparable to those induced by immunization with ri(E1$^-$).Ad26.dE3.Mos1Env, a replication-incompetent Ad26 vector expressing Mos1Env, which was previously evaluated (2, 7). To assess cellular immune responses, splenocytes were assessed by IFN-γ ELISPOT assays on day 28 postimmunization. Cellular immune responses generated by rcAd26.dE3.dE4.Mos1Env were comparable to those generated by the replication-incompetent vector ri(E1$^-$).Ad26.dE3.Mos1Env when stimulated with Mos1Env and PTE Env peptide pools, as shown in Fig. 6B. These data demonstrate that the rcAd26.dE3.dE4.HIVMos1Env vector is immunogenic in mice, although the potential benefits of vector replication were not assessed.

DISCUSSION

We have developed a replication-competent Ad26 vector platform for clinical evaluation. All of the rcAd26 vectors were attenuated compared with wild-type Ad26 in both the A549 and HuTu80 cell lines. The attenuation was most pronounced when both E3 and E4 were deleted and the HIV-1 Env transgene was expressed. Using quantitative PFU assays, the clinical candidate vector rcAd26.dE3.dE4.Mos1Env showed a 4.4-log reduction in replicative capacity compared with that of wild-type Ad26 in the duodenal cell line HuTu80. In addition, rcAd26.Mos1Env exhibited slower growth kinetics than that of rcAd4.H5, which has already been proven safe in a phase 1 clinical trial. The rcAd26.dE3.dE4.Mos1Env vector was immunogenic in mice, although the impact of vector replication could not be assessed, since rcAd26 does not replicate in mice. This vector is currently being evaluated in a phase 1 clinical trial (rcAd001/IAVI R001).

The deletion of E3 and E4 and the addition of the Mos1Env transgene all appeared to contribute to vector attenuation in a stepwise fashion, suggesting at least two mechanisms that may contribute to the attenuation of these vectors. First, there may be a requirement for E3 or E4 for optimal Ad26 replication, although this requirement was not evident with Ad5 vectors (22). Second, expression of the HIV-1 Env transgene likely reduces replication efficiency, as it requires substantial cellular metabolic resources.

A replication-incompetent Ad26 vector expressing a prototype HIV-1 EnvA antigen has already been tested in humans in three phase 1 clinical studies, and to date, it has been shown to be safe and immunogenic (3–5). However, replication-competent Ad26 has not been evaluated in humans. Ad26 is a biologically different less inflammatory vector than Ad5 and is different from Ad5 in terms of receptor usage, *in vivo* tropism, interactions with den-

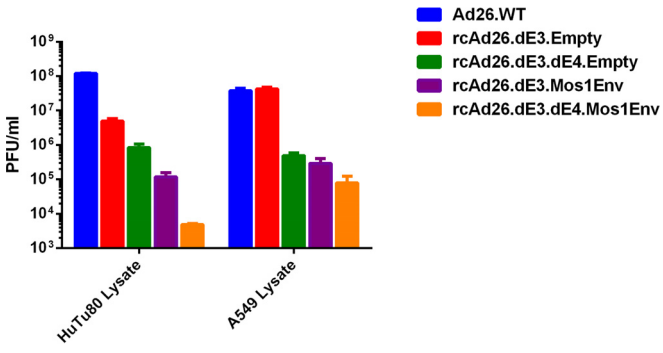

FIG 2 Titers generated by the panel of rcAd26 vectors (Table 1) were compared in lysates from cells infected with each vector at an MOI of 1,000 vp/cell and harvested at full CPE. Lysates from either infected HuTu80 cells (human duodenum; does not complement E1) or A549 cells (human; does not complement E1) were compared by performing the plaque assay in duplicate in A549 cells (human; does not complement E1). Data are shown as means with standard deviations.

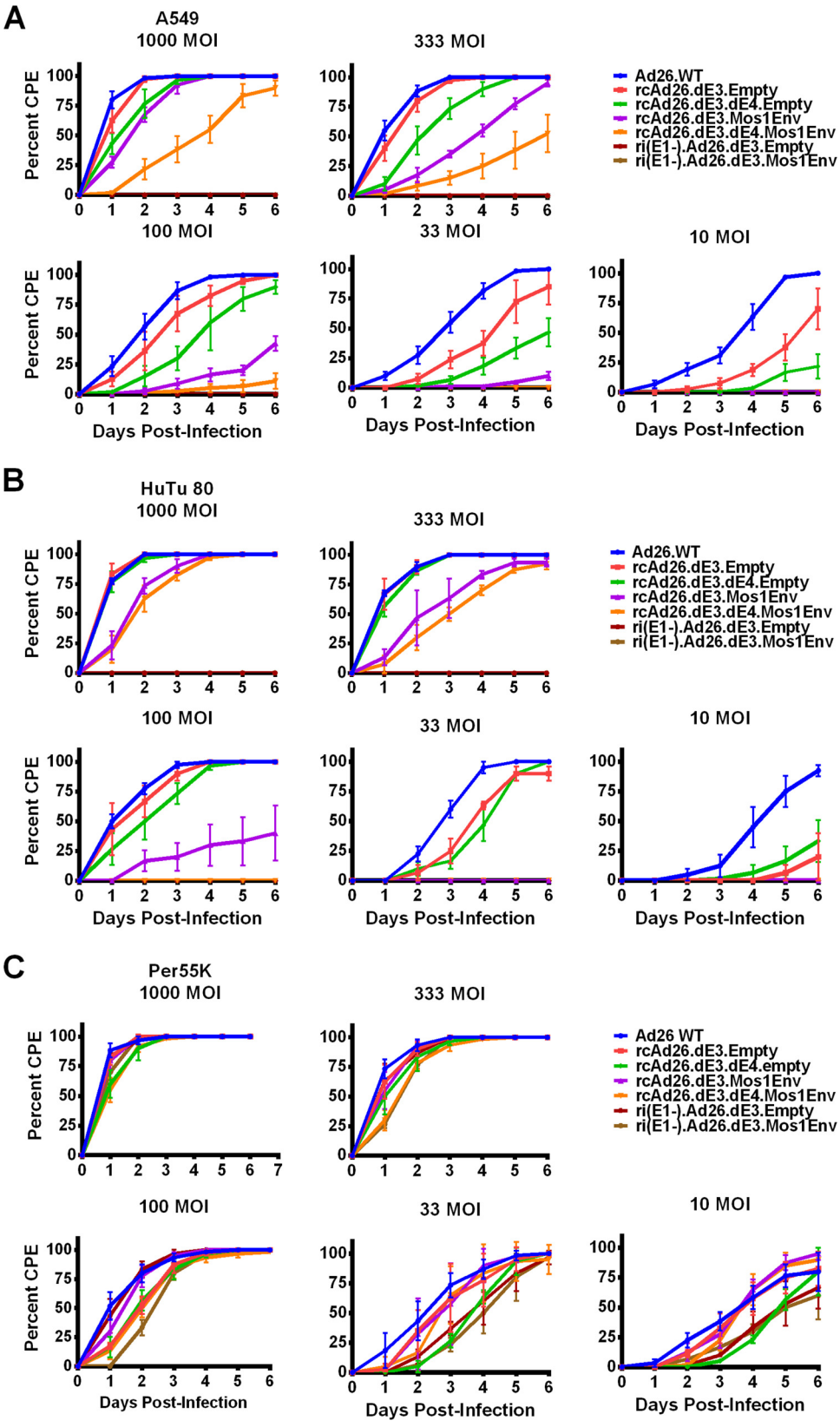

FIG 3 Growth of rcAd26.dE3.dE4.Mos1Env is attenuated *in vitro* compared to that of wild-type Ad26. The growth kinetics of a panel of Ad26-based vectors (Table 1) were compared *in vitro*. Cell lines were infected with vectors or wild-type virus at an MOI of 1,000, 333, 100, 33, or 10 vp/cell, and they were monitored daily for CPE over 6 days. Vector growth was evaluated in A549 cells (human; does not complement E1) (A), HuTu80 cells (human duodenum; does not complement E1) (B), and Per55K cells (human; complements E1) (C). Error bars indicate standard errors of the means (SEM).

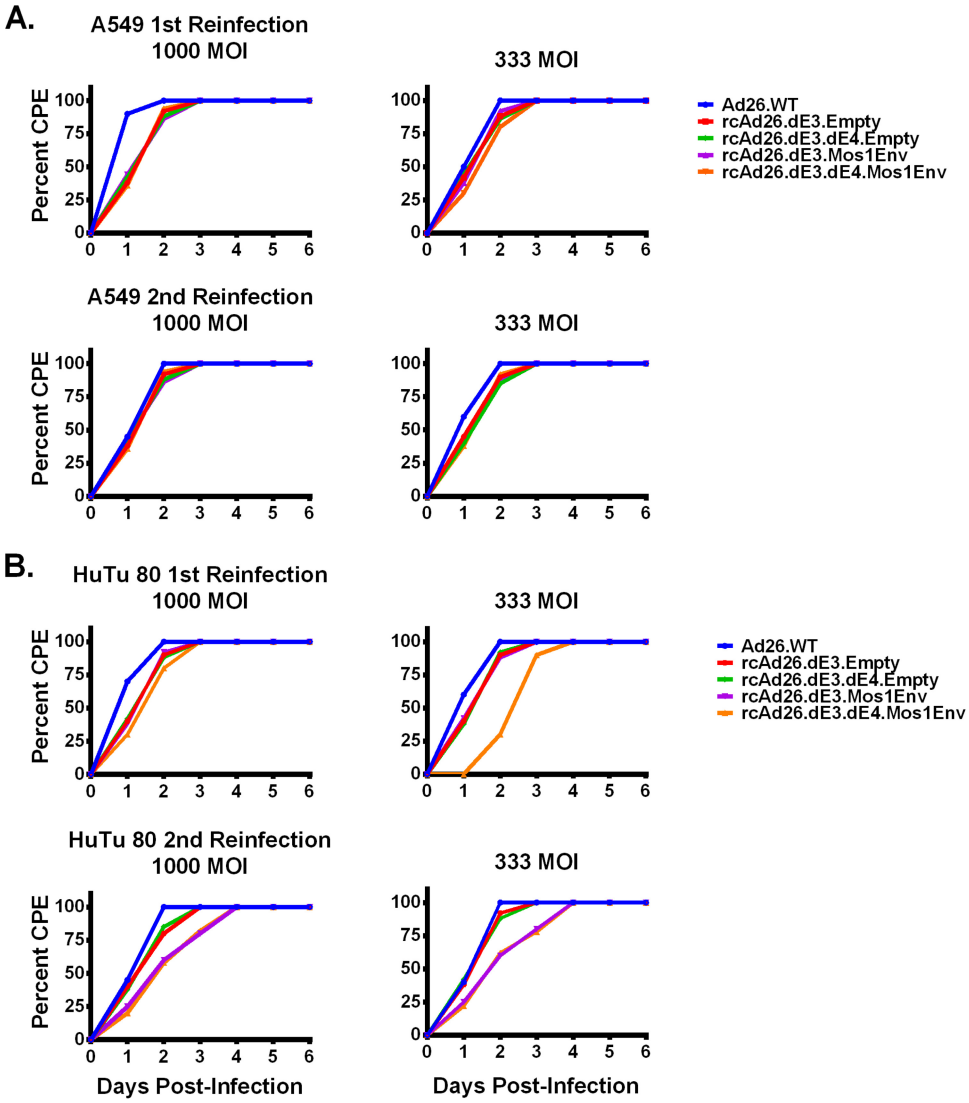

FIG 4 Replication of rcAd26 vectors is observed through two cell passages. The replication kinetics of the panel of rcAd26 vectors (Table 1) were compared through two passages in cell culture. Cell lysate was harvested from cultures at full CPE after infection at an MOI of 1,000 or 333 vp/cell. One hundred microliters of this lysate was used to infect either A549 (A) or HuTu80 (B) cells, depending on the original cell line used. The reinfected culture was harvested at full CPE, and 100 μl of these lysates was used to infect either A549 (A) or HuTu80 (B) cells with a second passage of vector. CPE was monitored for 6 days.

dritic cells, innate immune profiles, and adaptive immune phenotypes (16, 17, 23–29).

It remains unclear how to increase the potency and durability of HIV-1-specific immunity. One strategy is to use live replicating vectors (6). Live attenuated viral vaccines have been shown to be effective in both humans and animals (6) and include some of the most widely used licensed vaccines for measles-mumps-rubella viruses, yellow fever virus, varicella virus, influenza virus, and rotavirus (30). Attenuated simian immunodeficiency virus (SIV) has also shown robust protection against challenge with pathogenic SIV in rhesus monkeys (31–34), but live attenuated HIV-1 has not been pursued as a vaccine concept due to safety concerns (35). Recent studies have also shown that immunization with a replicating cytomegalovirus vector expressing SIV antigens led to immune control and possible clearance of highly pathogenic SIV infection in approximately half of rhesus monkeys (36). These data suggest the potential utility of replicating vectors expressing HIV-1 antigens.

The protective efficacy of replication-competent viral vectors may theoretically be superior to that of replication-incompetent viral vectors, as they might elicit different or more comprehensive innate, cellular, and humoral immune responses. In addition, replicating vectors may lead to enhanced mucosal immunity, although these hypotheses remain to be tested. These features are relevant to HIV-1 vaccine design, as HIV-1 exposure occurs at mucosal sites, and early HIV-1 replication targets mucosal $CD4^+$ T cells within gut-associated lymphoid tissue (GALT) (37). It is likely that protective immunity will require antibody responses and host $CD8^+$ T cells that can limit HIV-1 replication (38).

Live attenuated adenovirus vaccines have a proven track record of safety and efficacy for the prevention of Ad4 and Ad7 respiratory illness (39–41). These vaccines have been adminis-

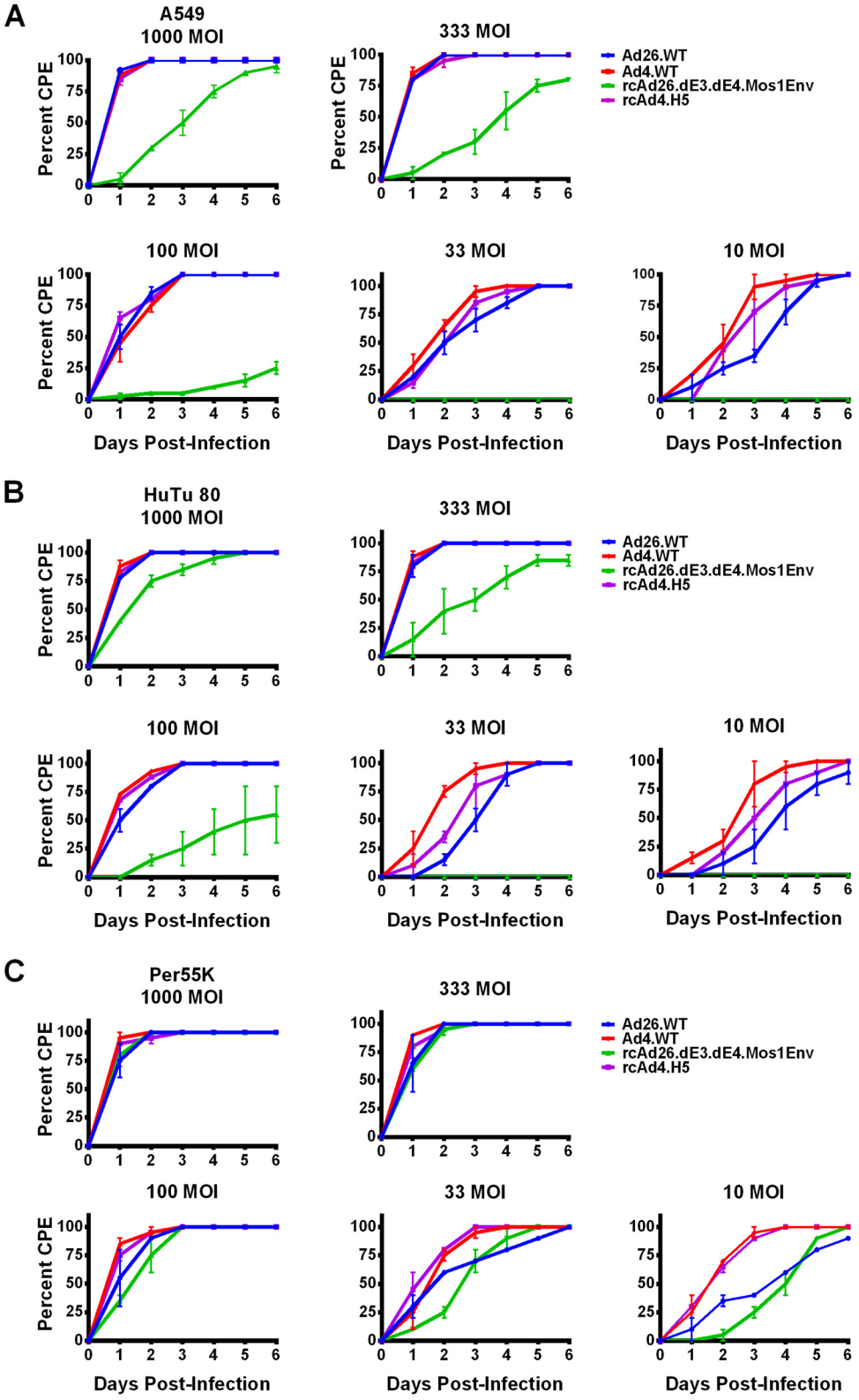

FIG 5 rcAd26.dE3.dE4.Mos1Env is attenuated *in vitro* compared to rcAd4.H5. The growth kinetics of a panel of Ad26-based and Ad4-based vectors (Table 1) were compared *in vitro*. Cell lines were infected with vectors or wild-type virus at an MOI of 1,000, 333, 100, 33, or 10 vp/cell, and they were monitored daily for CPE over 6 days. Vector growth was evaluated in A549 cells (human; does not complement E1) (A), HuTu80 cells (human duodenum; does not complement E1) (B), and Per55K cells (human; complements E1) (C). The growth of rcAd26.dE3.dE4.Mos1Env and rcAd4.H5 is shown. Error bars indicate standard errors of the means (SEM).

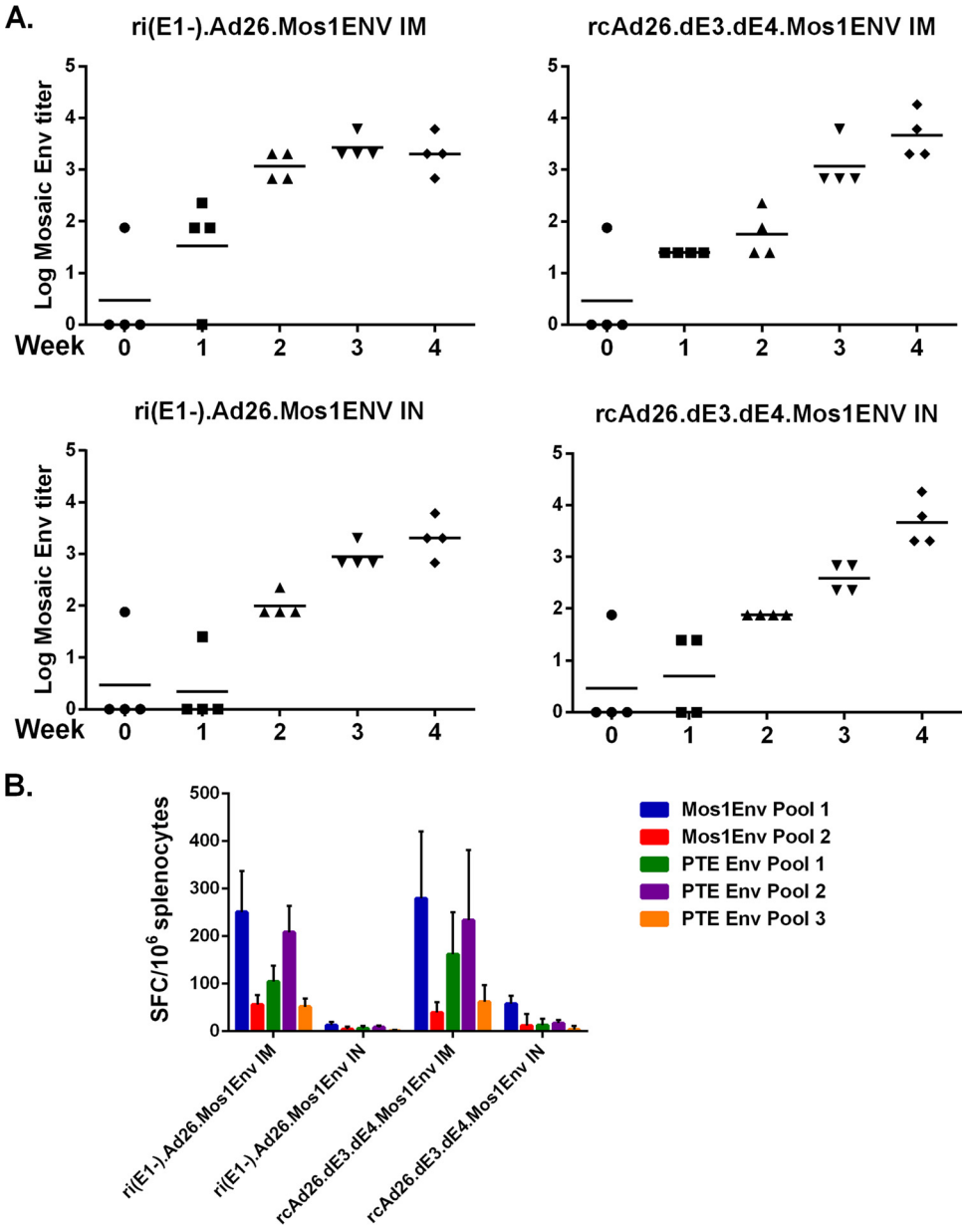

FIG 6 rcAd26.dE3.dE4.Mos1Env is immunogenic in mice. Humoral and cellular immune responses to rcAd26.dE3.dE4.Mos1Env and ri(E1$^-$).Ad26.dE3.Mos1Env were assessed in mice. BALB/c mice were immunized intramuscularly or intranasally with 1×10^{10} vp of each vector ($n = 4$). (A) Serum was taken on day 0 preimmunization and weekly until day 28 postimmunization, and antibody titers were assessed by ELISA using the HIV-1 Mos1Env protein. Data are shown as responses from individual mice. Horizontal lines indicate the means. (B) Cellular immune responses were determined by IFN-γ ELISPOT assays. Spleens were harvested on day 28 and stimulated with the Mos1Env 1, Mos1Env 2, PTE Env 1, PTE Env 2, and PTE Env 3 peptide pools. Data are shown as means with standard deviations.

tered safely to >10 million people over the span of nearly 60 years (41) and effectively prevent adenovirus disease outbreaks in military recruits (40). Oral live recombinant Ad4 (Ad4-H5-Vtn, or rcAd4.H5) vectors for influenza were also recently shown to be safe and immunogenic in an early phase clinical trial (14, 15). In addition, rcAd4 expressing HIV-1 envelope 1086 clade C is being evaluated (42). The lower replicative capacity of rcAd26.dE3.dE4.Mos1Env compared with that of rcAd4.H5 *in vitro* (Fig. 5) will facilitate, from a safety and regulatory perspective, the clinical development of rcAd26.dE3.dE4.Mos1Env, which is currently in a phase 1 clinical trial.

In this study, we describe the preclinical development of a replication-competent Ad26-based vaccine vector that expresses a mosaic HIV-1 Env immunogen. The rcAd26.dE3.dE4.Mos1Env vaccine candidate was found to be substantially attenuated compared to wild-type Ad26 as a result of vectorization, which has important clinical, regulatory, and immunologic implications.

ACKNOWLEDGMENTS

We thank P. Anklesaria, N. Russell, M. Pau, H. Schuitemaker, F. Ball, C. Bleckwehl, L. Parenteau, Z. Cama, and F. Stephens for generous advice and assistance.

We acknowledge support from the Bill and Melinda Gates Foundation (grant OPP1033091), the National Institutes of Health (grants AI060354, AI078526, AI084794, and AI096040), and the Ragon Institute of MGH, MIT, and Harvard.

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